THE VALUE OF AgNOR STUDY IN RADIOTHERAPY TREATED SQUAMOUS CELL CARCINOMA OF THE CERVIX - AS A DIAGNOSTIC AND PROGNOSTIC MARKER

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CERTIFICATE

This is to certify that this dissertation entitled **" THE VALUE OF AGNOR STUDY IN RADIOTHERAPY TREATED SQUAMOUS CELL CARCINOMA OF THE CERVIX - AS A DIAGNOSTIC AND PROGNOSTIC MARKER "** is the bonafide record work done by **Dr.D.SATHIYABAMA** submitted as partial fulfillment for the requirements of M.D. Degree Examinations Branch III Pathology, March 2007.

Dr.T.B.UMADEVI M.D.,
Professor & Head of the Department,
Department of Pathology,
Thanjavur Medical College,
Thanjavur.

THE DEAN
Thanjavur Medical College,
Thanjavur.

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CONTENTS

INTRODUCTION

Carcinoma Cervix is the second most common cancer worldwide especially in perimenopausal period. In developing countries like Asia, Africa and South America it is the commonest malignancy largely due to lower socioeconomic status, poor hygiene, illiteracy and paucity of screening programs.(14,15,51)

Pre – Cancerous lesions precedes a majority of cervical carcinoma and may exist in the non invasive stage for longer periods and shed abnormal cells which can be detected by exfoliative cytology using papanicoloau stain (2,14,15). Papanicoloau smears remain a simple screening method for detecting the precancerous lesions of cervix inspite of limitations due to sampling and preparation errors. With effectiveness of Papanicoloau cytological test and the accessibility of the cervix by colposcopic examination and biopsy, the detection rate of precancerous lesions have improved.

In invasive carcinoma of cervix, which are largely detected by biopsies, cancer cell proliferation greatly influences the clinical out come of the patients. The available evidences indicate that evaluation of cell kinetic parameters may help surgical Pathologists and Oncologist to define the biological behaviors of cancer.

A variety of tests are available for the assessment of cellular proliferative activity. Mitosis count has been the traditional method, however it detects only major differences and can be technically difficult and time consuming. With flow cytometry, the percentage of dividing cells

calculated as the S-Phase fraction. The main disadvantage is that specimen is destroyed in the process, thus cellular subpopulations within the tumour or admixtures of normal cells cannot be evaluated. Immunohistochemical methods for cell proliferation markers like Ki – 67, PCNA (Proliferating Cell Nuclear Antigen), DNA polymerases can be used, but they are more complicated and expensive than the silver stain used to show Nucleolar Organizing regions.(13,15)

A significant correlation between the interphase AgNOR (Argyrophilic Nucleolar Organizer Regions) quantity and prognosis has been reported in many neoplasms like Carcinoma breast, Lung, Gastrointestinal tract, and hematological malignancies (1,3,4,5,6,7,8,13,30). There was A CLOSE CORRELATION BETWEEN THE PROLIFERATIVE BEHAVIORS AND THE QUANTITATIVE AND QUALITATIVE CHANGE OF AgNOR (SILVER STAINED NUCLEOLAR ORGANIZER REGIONS) WAS FOUND IN OBVIOUSLY MALIGNANT NEOPLASMS.

There are also many studies using AgNOR conducted in preinvasive and invasive squamous epithelial lesions of cervix and AgNOR(20,22,2,3,24,27,28,31,32,34,46,48,51,52,54) proved to be a simple inexpensive and reliable proliferation marker in lesions of cervix.

Radiotherapy, which is the main mode of treatment in Carcinoma Cervix. AgNOR proved to be a sensitive marker to predict the prognosis of the patient treated with radiotherapy. In a rural and semi-urban areas where sophisticated methods are not available, AgNOR COUNTS IN CERVICAL SMEARS PROVE TO BE A SIMPLE AND COST EFFECTIVE METHOD IN THE DIAGNOSIS, PROGNOSIS AND FOLLOW UP OF CARCINOMA CERVIX PATIENTS.(16,21,26)

The QUANTITY OF INTERPHASE AgNOR (SILVER STAINED NUCLEOLAR ORGANIZER REGIONS) IS CONSIDERED AS A PARAMETER OF CELL KINETICS. Nucleolar Organizer

Regions (NOR) are components of ribosomal DNA located in the short arms of acrocentric chromosomes of 13,14,15,21 and 22 and transcribe to ribosomal RNA. NOR's vary in size and shape according to nucleolar transcription. They are intimately related to the cell cycle and may be RELATED TO PROLIFERATION AND PLOIDY.(1,7,18)

A prospective study of Carcinoma Cervix with AgNOR is undertaken to confirm the diagnosis before radiation to access the prognosis at consecutive intervals of 4 – weeks and 8 - weeks with special attention to the Nucleolar changes. In addition, the recent literature regarding Carcinoma Cervix and value of AgNOR in diagnosis and treatment of Carcinoma Cervix is also reviewed.

AIM OF THE STUDY

1) To evaluate the incidence and prevalence of Carcinoma Cervix.
2) To assess the value of **AgNOR IN CERVICAL CANCERS AS WELL AS IN CONTROL.**
3) To assess the **AgNOR SENSITIVITY ACCORDING TO DIFFERENTIATION AND MORPHOLOGICAL CHARACTERIZATION**.
4) To assess the **PROLIFERATIVE ACTIVITY OF NEOPLASTIC CELLS AND IT'S PROGNOSIS**.
5) To evaluate the **TREATMENT RESPONSE WITH PRE AND POST EXTERNAL BEAM RADIATION IN CERVICAL CANCER**.

MATERIALS AND METHODS

This study includes 50 Carcinoma Cervix patients referred from RAJA MIRASUDAR HOSPITAL which is affiliated to THANJAVUR MEDICAL COLLEGE, THANJAVUR during the two year period from JUNE 2004 to JUNE 2006 and 25 cases served as control.

A detailed clinical history like age, parity, age at menarche, years of married life, years after last delivery, hematological investigations, review of previous cytology and histopathological examination, family status/ socio economic status was done in all cases.

Cervical and Vaginal smears were collected from controls and from each patient prior to radiation therapy, using Wooden Ayres Spatula. By putting the woman on the examination table in dorsal position speculum was introduced into the vagina exposing the cervix and Wooden Ayres Spatula was introduced in to the posterior fornix. The Scraped material obtained was then spread on a slide. The pointed end of the Ayres Spatula was introduced into the cervical OS and rotated 360°, sampling the whole ectocervix. For each patient two slides were taken and they were fixed immediately with isopropyl alcohol.

The cyto brush was also used in those patients to whom it is difficult to get endocervical samples and those who have stenotic cervical OS especially in post menopausal women. A cyto brush was introduced into the cervical OS till few bristles were seen outside the cervical OS. The brush was rotated 180° and then the brush was rolled over on a glass slide and specimen was fixed immediately.

The Cervical and Vaginal smears collected from controls and from patients prior to radiation therapy were subjected to AgNOR staining.

TECHNIQUE:

Single step AgNOR staining technique was employed for the demonstration of AgNOR's (APPENDIX – 1). The freshly prepared solution was poured on to the smears which were then left in dark at 37°c for 30 minutes. Slides were dehydrated in 3 changes of acetone, cleared in Xylene and mounted in DPX.

The patients were given 40 – 50 gray of cobalt – 60 teletherapy divided in 20 – 25 fraction with daily fractions of 1.8 to 2.0 gray with five fractions per week.

In ideal patients, who respond to radiotherapy, as the tumor shrinks making the introduction of the uterine and vaginal applicators easy, the rest of the dose is delivered by brachytherapy by referring the patients to other higher centers.

The patients were followed and Cervical and Vaginal Smears were collected at intervals of 4 and 8 weeks after completion of radiotherapy. AgNOR counting was carried out as proposed by Chiu et all (5).

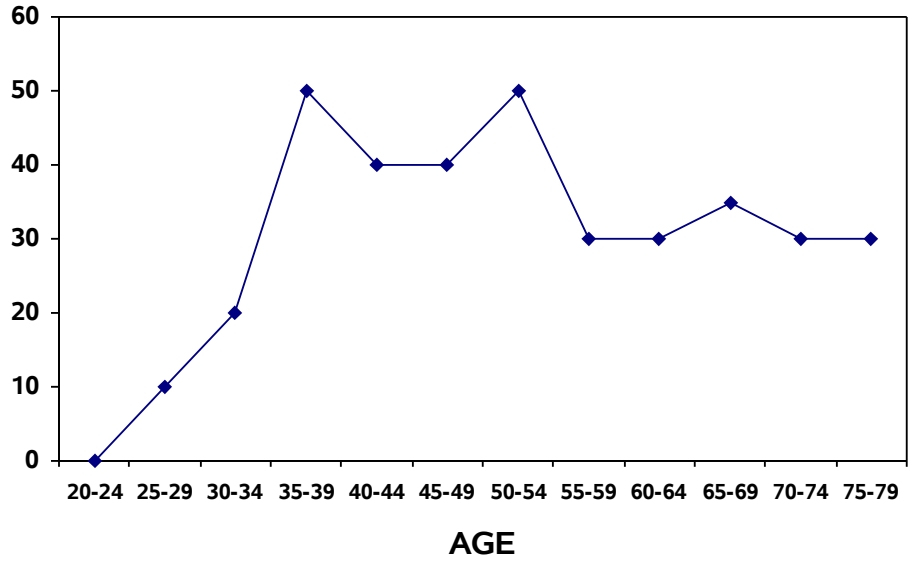
REVIEW OF LITERATURE

INVASIVE CARCINOMA OF CERVIX:

The cervix is the commonest site for female genital tract cancer; statistics vary considerably from country to country and from race to race. So in African and Asian women living in poor conditions, the incidence and relative mortality rate of the carcinoma cervix is 4-5 times higher than those seen in developed countries.(14,15,51)

AEITOLOGY:

Age: Although invasive cancer of cervix is reported at all ages, it has two peaks, one at about 35 yrs and another at 50-55 years, following which there is reduced incidence.(14,15,35,39)



Cervical Intra epithelial Neoplasia (CIN) occurs at a much lower age. One third of the cases being found in women less than 30 years old.(2,16)

Religion and Race:

The women of Orthodox Jews and in Muslims, the incidence of cervical cancer is less due to ritual circumcision done in Male Children at the childhood. Certain races it is unusually common, for example Africans they are susceptible even if they live in conditions of squalor.

Social and Economic Factors:

The people living in low Socio economic status are more prone to cervical carcinoma. The possible operating factors are low standards of cleanliness (including penile hygiene), coitus at an early stage, and frequency of sexual intercourse and promiscuity of both partners.

Coitus:

The sexually active women are 4-5 times more likely to develop cancer than sexually inactive women. It is postulated that smegma and spermatozoa are themselves carcinogenic.

Cervical Infection:

Human papilloma virus plays an important role in virtually all cases.

15-20 types of HPV have been associated with cervical carcinoma, type 16, 18, 31 and 45 accounts for 80% of cervical carcinoma. HPV 16 is associated with 50% of cases. (14,15,35,44)

Hormonal Factors:

There is some evidence that Oestrogen - Progesterone Oral Contraceptive pill usage favours CIN changes and also for Adeno carcinoma.

Predisposing Histological Status:

Certain histological changes in the cervix which are alleged to be “Pre cancerous” or which

are sometimes confused with cancer include basal oell hyperplasia, squamous cell metaplasia and CIN. Of these, only CIN II-III are likely to significant forerunners of invasive carcinoma.(26,50)

Histological Classification of Cancer Cervix:

Carcinoma cervix nearly always starts at the squamo-columnar epithelial junction and 80-90% cases are squamous cell in type.

In 5-10% of cases it is entirely columnar cell in pattern (AdenoCarcinoma) and the frequency is increasing especially in young women who are smokers or pill-users. The remainder constitutes the mixed types.

WHO Histological Classification Of Tumors Of The Uterine Cervix

Epithelial Tumors

Squamous tumors and precursors
- Squamous cell carcinoma, not otherwise specified
  - Keratinizing
  - Non – Keratinizing
  - Basaloid
  - Verrucous
  - Warty
  - Papillary
  - Lymphoepithelioma –like
  - Squamotransitional
- Early invasive (micro invasive) squamous cell carcinoma
- Squamous intraepithelial neoplasia
  - Cervical intraepithelial neoplasia (CIN) 3 /
  - Squamous cell carcinoma in situ
- Benign squamous cell lesions
  - Condyloma acuminatum
  - Squamous papilloma

- Fibroepithelial polyp

Glandular tumors and precursors

- Adenocarcinoma
  - Mucinous adenocarcinoma
    - Endocervical
    - Intestinal
    - Signet-ring cell
    - Minimal deviation
    - Villoglandular
  - Endometrioid adenocarcinoma
  - Clear cell adenocarcinoma
  - Serous adenocarcinoma
  - Mesonephric adenocarcinoma
- Early invasive adenocarcinoma
- Adenocarcinoma in situ
- Glandular dysplasia
- Benign glandular lesions
  - Müllerian papilloma
  - Endocervical polyp

Other epithelial tumors

- Adenosquamous carcinoma
  - Glassy cell carcinoma variant
- Adenoid cystic carcinoma
- Adenoid basal carcinoma
- Neuroendocrine tumors
  - Carcinoid
  - Atypical carcionoid
  - Small cell carcinoma
  - Large cell neuroendocrine carcinoma
- Undifferentiated carcinoma

Mesenchymal tumors and tumor – like conditions

- Leiomyosarcoma
- Endometrioid stromal sarcoma, low grade
- Undifferentiated endocervical sarcoma

Sarcoma botryoides
Alveolar soft part sarcoma
Angiosarcoma
Malignant peripheral nerve sheath tumor
Leiomyoma
Genital rhabdomyoma
Postoperative spindle cell nodule

Mixed epithelial and mesenchymal tumors

Carcinosarcoma (malignant müllerian mixed tumors, metaplastic carcinoma)
Adenosarcoma
Wilms tumor
Adenofibroma
Adenomyoma

Melanocytic tumors

Malignant melanoma
Blue naevus

Miscellaneous tumors

Tumors of germ cell type
Yolk sac tumor
Dermoid cyst
Mature cystic teratoma

Lymphoid and haematopoetic tumors

Malignant lymphoma (specify type)
Leukaemia (specify type)

Secondary tumors

Reagan et al (1957) recognized three groups of squamous cell carcinoma

- Large cell Keratinizing type
- Large cell Non – Keratinizing type
- Small cell Non – Keratinizing type

and other categories have been adopted by World Health Organization (Ritton & Christopherson 1973).

Cervical Intraepithelial Neoplasia:

This is an intraepithelial neoplasia that encompasses a continuum of morphologic changes arising in the basal layer of the stratified squamous epithelium of the transformation zone and extending to the entire thickness of the epithelium. The progression is divided into CIN I,II, and CIN III. Almost 60% of CIN I and 50% of CIN II lesions regress spontaneously. The remainder either persists or progresses through intermediate stages to invasive cancer. The majority of CIN III lesions progress to invasive cancer over 10-20 years. The majority of high-grade lesions are associated with HPV-16, 18, 31 and 33.(14,15,35)

Symptoms and Signs:

In its early stage, invasive carcinoma causes no symptoms and is discovered accidentally. Irregular uterine bleeding, post coital bleeding, foul smelling discharge with an offensive odour are present in most cases.

On examination per vaginum cervix shows indurations, irregularity and easily bleeds on touch.

Diagnosis:

Screening for prevention of cervical cancer

Cervical cytology with Papanicoloau Staining (APPENDIX 2) has been the mainstay of screening. The most effective method of obtaining a specimen is to use a cytobrush for the endocervical canal and scraping the ecto cervix with an Ayres spatula.(25,49)

Cytological screening has been established in most developed countries resulting in marked decrease in incidence of invasive cervical cancer.

The pre invasive-stage can be detected by the cervical smear test, which is simple, safe, in expensive and generally acceptable to women. All sexually active women are at risk and should be screened every year.

Cytological terminology of Cervical Precancer:

Papanicoloau's Classification was used for many years and his classification I to V have been adhered to until recently by some cytologists and gynecologists.[2,25,49]

Class I	-	Negative	-	Absence of atypical / abnormal cells
Class II	-	Negative	-	Atypical cells present but without abnormal features
Class III	-	Suspicious	-	Cells with abnormal features suggestive but not conclusive for malignancy
Class IV	-	Positive	-	Cells and cell clusters fairly conclusive for malignancy
Class V	-	Positive	-	Cells and cell clusters conclusive for Malignancy

WHO Classification:

Ritton and Christopherson used conventional histological terminology of mild, moderate and severe dysplasia and carcinoma in situ, as well as atypical metaplasia.

British Society for Clinical Cytology Terminology (BSCC):

BSCC's first working party on terminaly recommended the term dyskaryosis

Cytology	Expected histology
Mild dyskaryosis	CIN I (Mild dysplasia)
Moderate dyskaryosis	CIN II (Moderate dysplasia)
Severe dyskaryosis	CIN III (Severe dysplasia)

Bethesda System:

The 1988 Bethesda system for reporting cervical / vaginal cytologic diagnosis was published. The workshop agreed that the Papanicoloau classification was no longer appropriate and proposed the three essential components of cervical / vaginal smear report.(2,25,49)

The 1991 Bethesda System for Reporting cervical / vaginal cytologic diagnosis:

A) Statement of adequacy of the specimen

a) Satisfactory for evaluation when the specimen has correct identification with a minimum of pertinent clinical information and the smears contain an adequate number of well preserved and well visualized epithelial cells, including endocervical.

b) Satisfactory for evaluation but limited-by lack of pertinent clinical data or smears have an excess of blood, inflammatory cells, contaminants or are poorly fixed with air drying artifacts etc or contain no adequate endocervical or transformation zone cells.

c) Unsatisfactory for evaluation (Specify reason)

B) General Categorization:

(Optional, used to facilitate the triage of the specimens)

a) Within normal limits

b) Benign cellular changes: see descriptive diagnosis

c) Epithelial cell abnormality: See descriptive diagnosis

C) Descriptive Diagnosis:

a) Benign Cellular Diagnosis

1) Infection with organisms morphologically consistent with Trichomonas Vaginalis.
2) Fungal organisms consistent with candida species
3) Predominance of coccobacilli consistent with shift in vaginal flora
4) Bacteria consistent with Actinomyces sp.
5) Cellular changes associated with herpes simplex virus
6) others

b) Reactive changes associated with

1) Inflammation (includes typical repair)
2) Atrophy with inflammation (“atrophic vaginitis”)
3) Radiation and chemotherapy effect
4) Intra uterine contraceptive device
5) Other

c) Epithelial cell abnormalities

I) Squamous cell

a) Atypical squamous cells of undetermined Significance qualify (AGUS)

b) Low grade squamous intraepithelial lesion (SIL)

encampassing changes of HPV and CIN I

c) High grade squamous intraepithelial lesion (SIL)

encompassing changes of CIN II and CIN III

d) Squamous cell carcinoma

d) Glandular cell

1) Endo metrial cells cytologically benign in a post meno pausal

Woman

2) Atypical glandular cells of uncertain significance qualify

3) Endocervical adenocarcinoma

4) Endometrial adenocarcinoma

5) Extra uterine adenocarcinoma

6) Adenocarcinoma NOS

The Bethesda System includes a new term Squamous Intraepithelial Lesion (SIL) which is divided into two grades. Low grade SIL to include HPV and CIN I and High grade SIL for cells from CIN II and CIN III.

This division of precancerous lesions of the squamous epithelium into two grades instead of three is intended to improve reproducibility of reports of abnormal cervical cytology and to relate classification to the management of the patient. High grade SIL is an indication for excision or ablation of the abnormal tissue, whereas Low grade SIL may be followed up initially by cervical cytology alone.

Comparison of terminologies used for abnormal squamous epithelial cells in cervical cytology:

CIN Grade	WHO	BSCC	Bethesda
		Borderline	Atypia (ASCUS)
I	Mild dysplasia	Mild dyskaryosis	Low grade SIL
II	Moderate dysplasia	Moderate dyskaryosis	High grade SIL
III	Severe dysplasia Carcinoma in Situ	Severe dyskaryosis	High grade SIL
	Epidemoid Ca	Severe dyskaryosis	Squamous Carcinoma
		? invasive carcinoma	

The Cytology of CIN and invasive squamous cell carcinoma Dyskaryosis

The term dyskaryosis means literally “abnormal nucleus”. The morphological abnormalities seen in the nucleus of epithelial cells in cervical smears include a combination of any number of the following.

1) Disproportionate nuclear enlargement
2) Hyperchromasia
3) Bi and Multinucleation
4) Irregularity in form and outline
5) Abnormal chromatin pattern appearing as coarsening, stippling, formation of clumps or strands and sometimes as condensation beneath the nuclear membrane producing apparent irregularities in its thickness
6) Abnormalities of the number, size and form of nucleoli

1) Cytological findings of Mild Dyskaryosis / CIN I

- Disproportionate nuclear enlargement, the nucleus usually occupy less than half the area of the cell
- Nuclear hyperchromasia

- Abnormal nuclear pattern
- Irregularity of the nuclear membrane
- Multiple abnormal nuclei
- Cytoplasm reduced and usually thin
- Cell borders are usually angular

2) <u>Cytological findings of Moderate Dyskaryosis / CIN II</u>

- ϕ Disproportionate nuclear enlargement, the nucleus usually occupying between one half and two thirds of the total area of the cell
- ϕ Abnormal chromatin pattern, often more marked than in mild dyskaryosis
- ϕ Irregular nuclear membrane
- ϕ Nuclear hyperchromasia
- ϕ Multiple abnormal nuclei
- ϕ Cytoplasm reduced, may be thick or thin
- ϕ Cell borders may be angular / rounded

3) <u>Cytological findings of Severe Dyskaryosis and CIN III</u>

- o Disproportionate nuclear enlargement with the nucleus usually occupying at least two thirds of total area of the cell
- o Abnormal chromatin pattern
- o Nuclear hyperchromasia
- o Irregularity of nuclear membrane
- o Multiple abnormal nuclei
- o Cytoplasm markedly reduced abnormal maturation of cytoplasm including Keratinization
- o Cell borders smooth or angular

- Nucleoli prominent when CIN III is wide spread
- Bizarrely shaped cells sometimes including fibre cells

4) <u>Micro invasive and Invasive Squamous Cell Carcinoma</u>

Cytological features of invasive carcinoma:

Most cytologic specimens are obtained by direct scraping of the lesion. The most important sampling error that may lead to false negative interpretation is when the clinicians scrapes the bottom of the ulceration rather than its margins which will contain only debris, blood and inflammatory cells, mixed with a few hard to recognize degenerated cancer cells.

<u>Well Differentiated (Keratinizing) Squamous Cell Carcinoma</u>

They make up to 20% of invasive cervical squamous cell carcinoma

Cellular Morphology:

-The cancer cells shed singly and in clusters (55%) sheets (30%), or malignant pearl (2%) or in synticial form (13%)

-The shape of the cell is very pleomorphic which may be polygonal (55%), flat, round (10%), tadpole (10%), spindle (5%), irregular or pearl formation (10%)

-The size of the nuclei ranges from 2-10 times the size of normal squamous cells. The shape is round (30%), oval (20%) or irregular (40%) and varies with the shape of the entire cell (elongated) in spindle cells and round, irregular in polygonal cells

-The cytoplasm shows keratin deposition (40%) in concentric, perinuclear, ring like formations

-Background of the smear shows a large number of nonspecific

Inflammatory cells and cellular debris (Tumor diathesis)

Moderately differentiated (Nonkeratinizing large cells carcinoma)

70 % of the cervical cancers are of this type

Cellular Morphology:

- The numerous diagnostic cells are large either single (70%) or syncytial formation (30%). Epithelial sheets and pearl formations are absent. The cells vary in shape (round to polygonal)
- The cytoplasm is usually basophilic varies in amount and contain little mature keratin
- The nuclei are round or oval and less irregular, chromatin is coarse and irregularly clumped
- The eosinophilic nucleoli are often larger and more prominent than seen in well differentiated cancer cells
- The background of the smear shows severe tumor diathesis, containing large amounts of degenerate cellular debris red blood cells and protein deposits

Poorly differentiated squamous cell carcinoma

Based on their cellular morphology and ultra structures, some of these cancers are thought to be Neuroendocrine origin. They are often located toward the endocervical canal and represent 7% of invasive cervical carcinoma.

Cellular Morphology:

An abundance of diagnostic cells (>1000) usually found in the smear, which are usually single diagnostic cells.

The cells vary moderately in size and shape and are usually either round or oval. The delicately vacuolated cytoplasm of these cells is basophilic (80%), scanty and often seen as a narrow rim. Because of the fragility of this cytoplasm large number of “naked” malignant nuclei are usually present in the smear.

The nuclei are angular / oval shaped, with irregular chromatin clumps, multi nucleation is common. In clusters, the cells may overlap, with intercellular or slit like spaces.

- Pseudo cannabalism (birds eye appearance) is common
- Severe tumor diathesis is seen

HISTOLOGY:

In Micro invasive carcinoma, usually displays extensive CIN III, from which tongues and islands of neoplastic cells extend through the basement membrane in a spray like pattern of later as confluent invasion.

Well Differentiated Squamous Cell Carcinoma includes the presence of large islands of infiltrating tumor cells with intercellular bridges, epithelial pearl formation and obvious kernatinization of the cell cyto plasm.

Moderately Differentiated Squamous Cell Carcinoma show some evidence of a squamous origin, consisting of solid islands and smaller groups of polygonal pleomorphic cells, but kertinization and intercellular bridges are less obvious and pearl formation is not seen.

Poorly Differentiated Squamous Cell Carcinoma invades as sheets, islands and single cells often invoking a pronounced inflammatory reaction. No definite squamous features are seen and their origin may only be established with special stains to exclude mucin production and IHC for keratin expression with monoclonal antibodies.(14,15,35)

The Nucleolar Organizer Regions – A Review

Nucleolar Organizer Regions (NOR's) are DNA loops transcribing to ribosomal RNA's. In the human karyotype, NOR are secondary constrictions of metaphase chromosomes and are located in the short arms of the acrocentric chromosomes 13,14,15,21 and 22. In chromosome preparations, NOR can be clearly visualized using In Situ hybridization or by ammoniacal or formic acid silver nitrate staining. The silver stainability or NOR is due to the presence of a peculiar set of acidic proteins, which are highly argyrophilic.(3,37,38)

Structure and Function of Interphase NOR

The nucleolus is a well defined structural, functional domain of the cell nucleolus in which ribosomal genes are located and ribosomal biogenesis occurs. Mammalian cell nucleoli constantly exhibit apart from intranuclear chromatin, three main components.

1) The Fibrillar Centers, which appear as rounded structures of different size composed of very thin loosely, inter oven fibrils.
2) The Dense Fibrillar Component frequently located at the periphery of the fibrillar centers, which is composed of densely packed fibrils.
3) The Granular component composed of granules surrounding the fibrillar components.

The fibrillar centers plus the closely associated dense fibrillar component have been identified as the inter phase counterparts of metaphase NOR.(37,38)

Interphase NOR can be considered to represent structural-functional units for rRNA transcription. The two main enzymes controlling transcription RNA polymerase I and topoisomerase I have been clearly localized in the interphase NOR by ultra structural and immuno cytochemical investigations. The AgNOR proteins play a fundamental role in the control of rRNA transcription and processing. The two main proteins are called Protein C23 or Nucleolin (105kda) and protein B23 or Numatrin or Nucleophosmin (39kda).

Interphase NOR and Nucleolar Pathology:

The size and shape of the nucleoli is highly variable from cell to cell and in the same type of cell from the resting to proliferative state. Ultrastructural studies have shown that this variability is due to different distribution of the nucleolar components. A solitary large fibrillar center surrounded by a rim of ribonucleoprotein characterizes the very small nucleoli of resting lymphocytes. Whereas numerous small fibrillar centers, each surrounded by a rim of dense fibrillar component frequently intermingled with granules is characteristic of large nucleoli of phytohaemagglutinin stimulated lymphocytes.(37,38)

Until recently, a precise evaluation of nucleolar morphology, and its change during cell activation or transformation was impossible to be performed in routine cytopathology and histology at light microscopial level. Small nucleoli are frequently undetected and large nucleoli, independent of their size and shape are generally defined as “Prominent”.

In 1986, Ploton and co-worker applying the formic acid silver nitrate staining method for AgNOR proteins at the light microscopy level, succeeded in a very precise visualization of interphase NOR. By means of this procedure interphase NOR appear as well defined black dots evaluated by light microscopy, and each dot correspond to one interphase NOR as visualized by the electron microscope.(37,38)

How can Interphase AgNOR be quantified?

The method more frequently employed consisted of counting the number of interphase AgNOR per cell, evaluating the sample directly at the light microscope by carefully focusing throughout the section thickness at very high magnification (100 x oil immersion level) (5,7,8).

Though it is time consuming and subjective errors do occurs, the drawbacks are eliminated by the morphometric evaluation of the area of interphase AgNOR using a computer assisted image analyses.

Interphase AgNOR and Diagnosis of Malignancy:

Ploton and co-workers have demonstrated that malignant cells have a greater quantity of interphase AgNOR than the corresponding benign or normal cells and therefore evaluation of interphase AgNOR distribution may be useful for the diagnosis of malignancy.(37,38)

According to Crocker, interphase AgNOR quantity can be recommended as a suitable parameter for diagnosis of malignancy only in a few type of cancers. AgNOR values were found to be useful in differentiating Nevo cellular nevi and malignant melanoma, between infiltrating lymphocytes and Oat cell carcinoma deposits in bronchial material, between reactive mesothelial proliferation and mesothelioma, normal cirrhotic and carcinomatous liver, between benign and malignant salivary gland tumors and between low grade and high grade Non Hodgkins Lymphoma. (6,7,8)

Therefore AgNOR quantify can be considered only as one, among the other well established cyto-histopathological parameters to be used for diagnosis of malignancy.

Nevertheless, it is incontestable that in a broad sense, the quantify of interphase AgNOR is greater in cancer than in normal or hyperplastic cells.

Why do Cancer Cells have a large number of interphase AgNOR?

Considering the central role of interphase AgNOR in rRNA synthesis, changes of interphase NOR distribution in cancer cells might be the consequence of the increased demand for ribosomal biogenesis which characterizes dividing cells.

In cells stimulated to proliferate, concurrently with an increased synthesis of AgNOR proteins, a progressive dispersal of ribosomal chromatin has been described, whereas in resting cells ribosomal sequences are located in highly compact structured chromatin.

INTERPHASE AgNOR AND CANCER PROGNOSIS

The data reported above concerning the relationship between interphase AgNOR and cell proliferation clearly indicate that the quantity of interphase AgNOR's can be considered as a parameter of cell kinetics. The greater the interphase AgNOR amount, the shorter the cell doubling time.(2,9,14)

The cell kinetic parameters may help pathologists and clinical oncologists to define the biological behaviors of cancer lesions, obtain valuable prognostic information and indications about specific treatments of cancer.

Neverthless, a significant correlation between interphase AgNOR quantity and patient survival has been reported in many types of neoplastic diseases.(34,41) In two types of cancer lesions interphase AgNOR evaluation has been proved to be of great importance.

1) Patients with lower number of interphase AgNOR in colorectal cancers still alive after 5 years, than those who presented with higher counts.

2) In stage I endometrical carcinoma, 10 year follow up study showed that 90% of patients with low interphase AgNOR values were still alive, whereas 60% of patients with high interphase AgNOR values died of the disease.

Multivariate analysis of survival in both these cancers demonstrated that quantity of AgNOR represented the most important prognostic parameter when compared to histological grade.

Interphase AgNOR quantification appears to be very interesting and promising method for the routine evaluation of cell kinetics for prognostic purposes. It is the only method which permits information to be obtained on the rapidity of cell proliferation in routinely processed samples. Only flow cytometry, by simultaneous analysis of DNA content and incorporation of injected bromo deoxyuridine in vivo, permits measurement of cell doubling time.

Interphase AgNOR staining is not expensive and very rapidly executed on the cytological smear and tissue sections. Moreover AgNOR distribution in cancer tissues can be directly perceived at light microscopic examination without using additional instruments for objective AgNOR quantification.(34,39,41)

RADIATION FOR CERVICAL CANCERS:

All cervical cancers, except stage I and II A are primarily treated with radiation therapy. The chief modalities of present day radiation treatment of cancer cervix are external photon beam treatment and brachytherapy.

External Beam Radiotherapy (EBRT) (Teletherapy):

EBRT is used to treat the pelvic lymphnodes, commoniliac lymphnodes, Para-aortic nodes and lateral parametria. A dose of 45-50 gy is delivered by EBRT over five weeks, with daily fraction of 1.8 to 2 gy, five fractions per week. As the tumor shrinks making introduction of uterine and vaginal applicators easy, rest of the dose is delivered by brachytherapy.

Brachytherapy:

The word “Brachy” in Greek means “short distance”. It implies placing radioactive sources in contact with or very close to target tissue. The implants are classified into High Dose Rate (HDR) and Low Dose Rate (LDR). Dose rates of 0.4 to 2 gy/hour are delivered in LDR brachytherapy so that the treatment time is 24-72 hours. In HDR brachytherapy implant uses dose rates > 12 gy/hour and the total dose can be delivered in few minutes.

Isotopes used in LDR brachytherapy are Cs^{137} (caesium) and for HDR brachytherapy Co^{60} (cobalt), Ir^{192} (Iridium) isotopes are used both for LDR and HDR therapy.(51)

OBSERVATION AND RESULTS

The prospective study included 50 exfoliative cytology specimens of clinically evaluated and diagnosed cases of squamous cell Carcinoma of Cervix. This study also includes 25 control specimens for comparison and correlation of efficacy of AgNOR in cervical cancers. The clinical, cytological feature, histopathological data and AgNOR study conducted in cases are listed in the master chart. *Table-1* shows the incidence of cervical cancers, referred for cytological evaluation and subsequent confirmation by histopathological examination, during the period from JUNE 2004 TO JUNE 2006.

TABLE-1

Sl.No	**Period**	**Total No. of Pap smears**	**Positive cases**
1	June 2004 – December 2004	280	10
2	January 2005 – June 2005	1201	15
3	July 2005 – December 2005	396	13
4	January 2006 – June 2006	286	12
Total			**50**

Table 1-A shows incidence of cervical cancers of biopsy / gross specimens referred for

histopathology evaluation after precytological evaluation (in proportion of cases).

TABLE 1-A

Sl. No	Period	Total No. of Gynaec Specimens	No. of Cervical Biopsy for cacx	Nonspecic changes	CIN change	Invasive squamous cell carcinoma		
						Well Diff SCC	Mod. Diff SCC	Poorly Diff SCC
1	June 2004– Dec 2004	934	205	31	29	42	101	2
2	Jan 2005 – June 2005	807	214	86	22	14	100	2
3	July 2005 – Dec 2005	872	208	71	12	9	115	1
4	Jan 2006 – June 2006	779	207	60	26	16	104	1
TOTAL = 3392						**Total Invasive Carcinoma**		**= 507**

AGE INCIDENCE:

The patients initially diagnosed with Papanicoloau Stain as Cervical Cancer were divided into 6 groups according to age (i.e., 20-30 yrs, 31-40 yrs, 41-50 yrs, 51-60 yrs, 61-70 yrs, and 71-80 yrs). There was increased incidence of cervical cancer observed in the age group of 41-50 years (38%) followed by 51-60 years (30%) and 31-40years (20%).

The age distribution of cervical cancer is given in the following *Table-2*.

TABLE-2

Sl.No	Age Group	No. of Cases	Percentage
1	20 – 30 years	2	4
2	31 – 40 years	10	20
3	41 – 50 years	19	38
4	51 – 60 years	15	30
5	61 – 70 years	3	6
6	71 – 80 years	1	2
TOTAL		**50**	**100**

Most of the patients referred for Papanicoloau stain were belonging to surrounding villages of low socioeconomic status with poor hygiene and lived in over crowded surrounding. Most of them presented with foul smelling discharge / post coital bleeding, bleeding PV for which initial Papanicoloau cytological examination was undertaken.

In all 50 cases of initially diagnosed cervical cancers with cytology further cervical biopsies were undertaken and histopathological examination was done.

The following *Table-3* shows distribution of cervical cancers according to differentiation.

TABLE-3

Sl.No	Grade	No. of Cases	Percentage
1	Well differentiated squamous cell carcinoma	9	18%
2	Moderately differentiated squamous cell carcinoma	37	74%
3	Poorly differentiated squamous cell carcinoma	4	8%

In this study most of the cases are Moderately differentiated (37 cases, 74%), **fig-2** followed by Well differentiated squamous cell carcinoma (9 cases, 18%) **fig-1**. Poorly differentiated carcinoma constitute only 8% of total cases **fig-3**.

The following *Table-3A* shows classification of cervical cancers (squamous cell carcinoma) according to morphological subtypes.

TABLE-3A

Sl.No	Morphologic Subtype	No. of Cases	Percentage (%)
1	Non Keratinizing SCC	420	82.8
2	Keratinizing SCC	75	14.8
3	Small Cell SCC	6	1.2
4	Basaloid SCC	1	0.2
5	Verrucous SCC	1	0.2
6	Warty SCC	1	0.2
7	Papillary SCC	2	0.4
8	Lymphoepithelioma Like	1	0.2

In 507 cases of cervical cancers , most of the cases are Non Keratinizing SCC (420 cases), Keratinizing SCC (75 cases), Small cell (6 cases), Basaloid SCC 1 case) **fig-4,4a,** Verrucous SCC (1 case) **fig-5,5a,** Warty SCC (1 case) **fig-6,6a,** Papillary SCC(2 cases), Lymphoepithelioma Like (1 case) **fig-7,7a** were referred for radiotherapy of which 50 cases were selected for our prospective study in which radiation was given and smears taken at 4 weeks and 8 weeks.

The Table-3B shows most of the cases are Non Keratinizing squamous cell carcinoma (35 cases, 70%) followed by Keratinizing squamous cell carcinoma (8 cases, 16%). Small cell, non keratinizing / Poorly differentiated / Neuro endocrine cancer constitute only 10% (5 cases), Papillary squamous cell carcinoma 4% (2 cases) during the prospective study period.

TABLE- 3B

Sl.No	Morphologic Subtype	No. of Cases	Percentage (%)
1	Non Keratinizing SCC	35	70
2	Keratinizing SCC	8	16
3	Small Cell SCC	5	10
4	Verrucous SCC	-	-
5	Warty SCC	-	-
6	Papillary SCC	2	4
7	Basolid SCC	-	-
8	Lymphoepithelioma Like	-	-

In this study 35 cases of Non Keratinizing squamous cell carcinoma, 8 cases of Keratinizing squamous cell carcinoma, 5 cases of small cell Non Keratinizing squamous cell carcinoma, 2 cases of Papillary variant of squamous cell carcinomas were subjected for further management to radiotherapy.

Evaluation of AgNOR counts in controls and in cancer cervix, AgNOR size variation and distribution was recorded according to the criteria provided by Ashan et all. Students't' test was applied for the statistical analysis of results.

Size variation was graded as follows

0 - More or less uniform size

1+ - Two different sizes

2+ - More than 2 different sizes (but not those of 3+)

3+ - All grades and sizes heterogenous

Distribution of AgNOR's in the nucleoli were graded

0 - Limited to nucleoli

1+ - Occasional dispersion outside the nucleoli

2+ - Moderate dispersion outside the nucleoli

3+ - Widely dispersed throughout the nucleus

The following *Table-4* shows the comparison of AgNOR counts, size variation, and distribution in control cases (25 cases) **Fig-8**.

TABLE-4

Sl.No	Group	AgNOR count / cell		AgNOR size Variation		AgNOR distribution	
		Range	Mean	0 - 1+	2+ - 3+	0 – 1+	2+ - 3+
1	Control-25 cases	1 – 3	1.63	22	3	23	2

22 cases exhibit size variation of 0 to 1+ and 3 cases shows 2+ to 3+ size variation. The AgNOR distribution is also 0 to 1+ in 23 cases and 2 cases shows 2+ to 3+. The AgNOR count per cell in all 25 cases ranged 1-3 AgNOR dots/cell, with mean of 1.63.

Similarly the comparison was done in 50 cases of Carcinoma Cervix diagnosed by histopathological examination prior to radiation as given in the following *Table - 4A*.

TABLE - 4A

Sl.No	Grade	AgNOR count		AgNOR size variation		AgNOR distribution	
		Range	Mean	0 - 1+	2+ - 3+	0 - 1+	2+ - 3+
1	Well differentiated SCC	3-8	3.76	2	7	1	8
2	Moderately differentiated SCC	3-5	3.84	3	34	2	35
3	Poorly differentiated SCC	3-9	4.12	-	4	-	4

In well differentiated squamous cell carcinoma (9 cases), the AgNOR count/cell ranged from 3-8 cell, with mean of 3.76, AgNOR size variation was 2+ to 3+ (7 cases), and 2 cases show 0 – 1+, AgNOR distribution ranges 2+ - 3+ (8 cases), one case 0 - 1+. **Fig-10**

In Moderately differentiated squamous cell carcinoma (37 cases), the AgNOR count/cell ranged 3-5/cell with mean of 3.84, AgNOR size variation was 2+ to 3+ in 34 cases and 2 cases show 0 to 1+, AgNOR distribution ranges 2+ to 3+ in 35 cases , two cases 0 to 1+. **Fig-14**

In Poorly differentiated squamous cell carcinoma (4 cases), the AgNOR count/cell ranged from 3-9/cell with mean of 4.12, AgNOR size variation and distribution ranged 2+ to 3+ in all four cases. **Fig-18**

Similarly comparison was done in 50 cases of carcinoma cervix, after 4 weeks of radiation as given in following Table -4B.

TABLE -4B

Sl.No	Grade	AgNOR count		AgNOR size variation		AgNOR distribution	
		Range	Mean	0 - 1+	2+ - 3+	0 - 1+	2+ - 3+
1	Well differentiated SCC (9cases)	2-6	3.2	6	3	6	3
2	Moderately differentiated SCC (37cases)	2-4	2.82	34	3	34	3
3	Poorly differentiated SCC (4cases)	2-5	3.35	2	2	2	2

In well differentiated squamous cell carcinoma (9 cases), the AgNOR count/cell ranged from 2-6 cell, with mean of 3.2. AgNOR distribution ranges 0-1+ in 6 cases, 2+ - 3+ in 3 cases, AgNOR size variation ranges from 0 – 1+ in 6 cases and 2+ to 3+ in 3 cases. **Fig-11**

In Moderately differentiated squamous cell carcinoma, the AgNOR count/cell ranged 2-4/cell with mean of 2.82, AgNOR size variation ranged 0 to 1+ in 34 cases, only 3 cases ranged 2+ to 3+, AgNOR distribution ranges 0 to 1+ in 34 cases and only 3 cases ranged 2+ to 3+. **Fig-15**

In Poorly differentiated squamous cell carcinoma (4 cases), the AgNOR count/cell ranged from 2-5/cell with mean of 3.35. AgNOR size variation ranged 0 – 1+ in 2 cases, 2+ to 3+ in 2 cases. AgNOR distribution ranged 0 to 1+ in 2 cases and 2+ to 3+ in 2 cases. **Fig-19**

Similarly comparison was done in 50 cases of carcinoma cervix, after 8 weeks of radiation as given in *Table -4C*.

TABLE -4C

Sl.No	Grade	AgNOR count		AgNOR size variation		AgNOR distribution	
		Range	Mean	0 - 1+	2+ - 3+	0 - 1+	2+ - 3+
1	Well differentiated SCC (9cases)	2-5	2.67	6	3	6	3
2	Moderately differentiated SCC (37cases)	2-3	2.19	34	3	34	3
3	Poorly differentiated SCC (4cases)	2-4	2.97	2	2	2	2

In well differentiated squamous cell carcinoma (9 cases), the AgNOR count/cell ranged from 2-5/cell with a mean of 2.67, the AgNOR size ranged 0 to 1+ in 6 cases, 2+ to 3+ in 3 cases. The AgNOR distribution ranged 0 to 1+ in 6 cases and 2+ to 3+ in 3 cases. **Fig-12**

In Moderately differentiated squamous cell carcinoma (37 cases), the AgNOR count/cell ranged from 2 to 3/cell with mean of 2.19. AgNOR size variation and distribution ranged 0 to 1+ in 34 cases, 3 cases ranged 2+ to 3+. **Fig-16**

In Poorly differentiated squamous cell carcinoma (4 cases), the AgNOR count/cell ranged from 2-4/cell with a mean of 2.97. AgNOR size variation and distribution ranged 0 to 1+ in 2 cases, 2+ to 3+ in 2 cases. **Fig-20**

DISTRIBUTION OF AgNOR'S IN CARCINOMA CERVIX PRE AND POST RADIATION THERAPY (RT)

TABLE-5

Sl.No	Category	No. of Cases	AgNOR Count/cell	
			Range	Mean
1	Control	25	1-3	1.63
2	Prior to RT	50	3-8	3.98
3	Post RT a) 4 weeks b) 8 weeks	 50 50	 2-5 2-4	 2.92 2.43
4	Persistence of Malignancy	8	3-6	3.54

1:2	P< 0.001	1:3a	P< 0.001	1:3b	P< 0.001	1:4	P< 0.001
2:3a	P< 0.001	2:3b	P< 0.001	2:4	P> 0.05	3a:4	P< 0.001
3b:4	P< 0.001						

AgNOR counts in control group ranged from 1-3 with a mean of 1.63. In all the 50 cases of carcinoma cervix prior to radiation therapy the AgNOR counts ranged from 3-8 with a mean of 3.98, significantly higher than that in the control group (P< 0.001). After irradiation a significant decline was noted in AgNOR counts which varied from 2-5 with a mean of 2.92 after 4 weeks of irradiation and from 2-4 with a mean of 2.43 after 8 weeks of irradiation. The AgNOR score in 8 patients ranged from 3-6 (Mean 3.54) which is significantly higher (P<0.001) than the values obtained from the control group and from the patients who showed favourable response to radiotherapy consistent with persistence of malignancy **Fig-21,22.** The AgNOR counts observed 4 and 8 weeks after radiotherapy compared shows significant reduction (P< 0.001) in the counts statistically.

DISCUSSION

Carcinoma Cervix accounts for 80% of all gynecological cancers including breast cancer being commonest cancer in women in the developing countries. The most common cancer among women in India is Cervical Cancer. The American cancer society estimates that 9,710 women will be diagnosed with and 3700 women will die of cancer of the cervix in 2006, however although globally, 5, 00,000 new cases of carcinoma cervix are recorded of which 1, 00,000 are from India. (15,42)

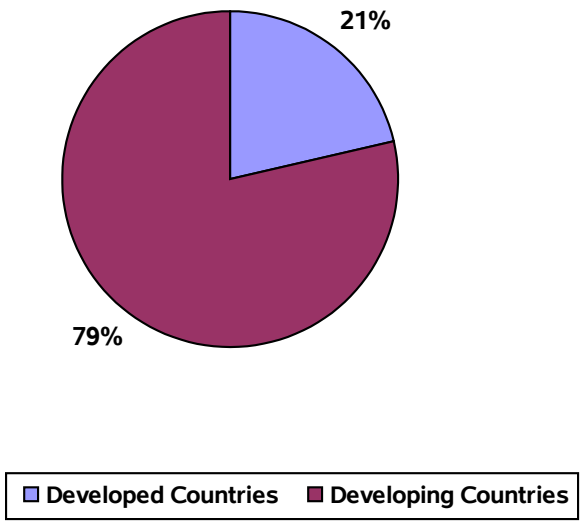
In our prospective study the average incidence of cervical cancer in biopsy specimen is 60.8% which is high when compared with studies conducted by various authors and literature.

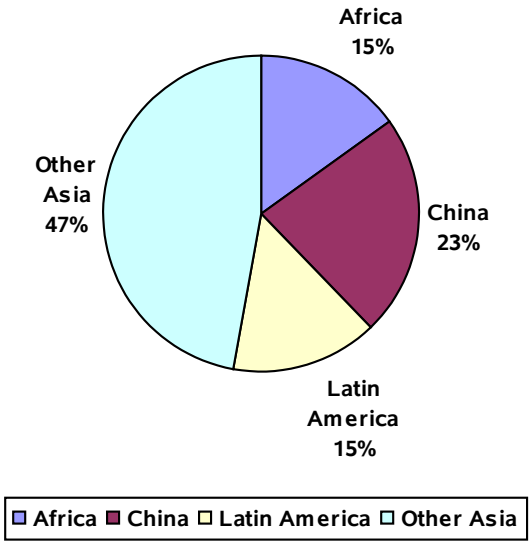
Present Study Incidence

TABLE -1A

Sl. No	Period	Total No. of Gynaec Specimens	No. of Cervical bx for cacx	Nonspecic changes	CIN change	Invasive squamous cell carcinoma		
						Well Diff SCC	Mod. Diff SCC	Poorly Diff SCC
1	June 2004– Dec 2004	934	205	31	29	42	101	2
2	Jan 2005 – June 2005	807	214	86	22	14	100	2
3	July 2005 – Dec 2005	872	208	71	12	9	115	1
4	Jan 2006 – June 2006	779	207	60	26	16	104	1
TOTAL = 3392						**Total Invasive Carcinoma = 507**		

The following Pie Chart shows the burden of cervical cancer all over the world.

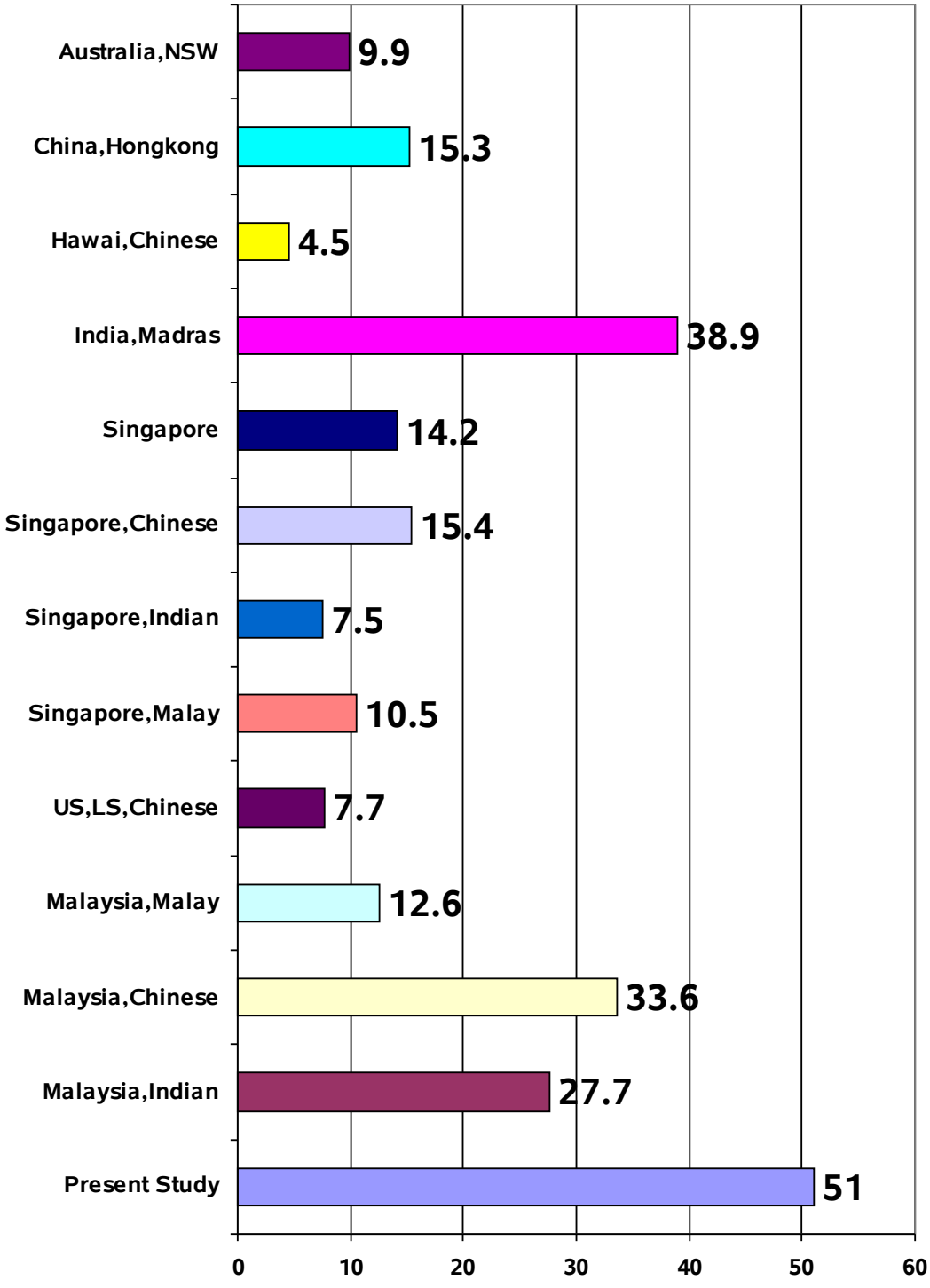




Estimated number of new cervical cancer in the year 1985

(Ref: Source Parkin et al 1993)

When considering the Age specific cancer incidence per 1, 00,000 population, the below bar chart shows that our study clearly shows increased ratio 51 / 1,00,000 population well comparable to the incidence data of India/Madras and Indian and Chinese people living in Malaysia and is well compared with various research workers as well as program like SEER (Surveillance Epidemiology and End Results) program.



In India, carcinoma cervix continues to lead the list of cancers afflicting female genital tract because of poverty, poor socioeconomic status, wide spread ignorance, poor personal hygiene, early marriage and child bearing and high parity status continue to prevail in our country. Religious taboos and social traditions instill a false sense of modesty which inhibits women, particularly from rural backgrounds from seeking medical aid for what they consider a minor gynecological problem.

The average age of women newly diagnosed with cervical cancer is between 50 and 55 years (14,15,35,36,40). This cancer rarely occurs in girls younger than 15. It begins to appear in women in their twenties. Cervical cancer is different from most cancers that tend to occur more often as people get older. Although cervical cancer does affect young women, many older women do not realize that their risk of developing cervical cancer does not go down as they age and that it is important for them to continue having Pap tests.

SEER Incidence:

From 2000-2003, the median age at diagnosis for cancer of the cervix uteri was 48 years of age. Approximately 0.1% were diagnosed under age 20. 15.4% between 20 and 34, 26.4% between 35 and 44, 23.3% between 45 and 54, 14.9% between 55 and 64, 10.4% between 65 and 74, 6.9% between 75 and 84, and 2.6% 85+ years of age.(16,51)

The age adjusted incidence rate was 8.8 per 1, 00,000 women per year. These rates are based on cases diagnosed in 2000-2003 from 17 SEER (Surveillance Epidemiology and End Results) geographic areas.

Incidence rates by race were

Race / Ethnicity	Women
All Races	8.8 per 1,00,000 women
White	8.5 per 1,00,000 women
Black	11.5 per 1,00,000 women
Asian/Pacific Islands	8.2 per 1,00,000 women
American Indian/Alaska Native+	7.2 per 1,00,000 women
Hispanic	14.2 per 1,00,000 women

In our study the youngest age group at which cervical cancer identified is 28 and 29 years and incidence is common between 41-50 years followed by 51-60 years(42,49). Our study contrast with other studies that the initial age of cervical cancer is 5-10 years earlier than the western population.

Among the cervical carcinomas, 80% are in advanced stages, i.e., stage II B, III and IV and only 20% are in early stages i.e., in stage I and II A, while in UK 90% cases are in early stage and only 10% are in advanced stages.

TABLE -6 **Patient Distribution According To Stage**

	Patients	Stage I B	II A	II B	III A	III B	IV A
Study Burdwan Medical College	Number (292)	14	18	98	14	142	8
	%	4.76	6.12	33.34	4.76	48.30	2.72
Present Study	Number (50)	5	8	29	1	2	5
	%	10	16	58	2	4	10

The *Table-6* shows that in our present study most of the patients are in advanced stage as study conducted in Burdwan Medical College, Bengal, a rural teaching hospital. Stage II B and above constituted > 70% and < 30% were in stages

of I B and II A which is well correlated. Most of the patients were coming for the first time in an already advanced stage; however the picture is reverse in western population, since females are evaluated consequently from the age of 18 years with Pap smears.

In our study above 74% of the cases are Moderately differentiated squamous cell carcinoma followed by 18% Well differentiated carcinoma. This distribution is well in correlation with various studies that Moderately differentiated infiltrating squamous cell carcinoma being the commonest type all over the world followed by Well differentiated squamous cell carcinoma when considering the morphological subtypes also, Nonkeratinising squamous cell carcinoma predominates (319 cases, 88.12% in total cervical cancers, 35 cases, 70% in the present study).

In this study evaluation of Cervical Cancer with the AgNOR status is done with Pap smears after initial diagnosis with histopathological examination, 4 weeks after radiotherapy, as well as 8 weeks after radiotherapy cytological assessment has become a standard procedure for early detection of squamous cell carcinoma per se, and for assessment of radiation therapy changes and for AgNOR scoring.

The following *Table-7* shows AgNOR count/cell in squamous cell carcinoma cervix, a comparison with other study groups and present study.

TABLE-7

Sl.No	Study Group	Grade of SCC	AgNOR Count/Cell	
			Range	Mean
1	Ghazala Mehdi & Kafl Aktar	o Well o Mod o Poor	3.7–3.77 3.75-3.89 3.86-4.2	3.74 3.82 4.03
2	Jyotima Agarwal & J.K Gupta	o Well o Mod o Poor		5.27 5.41 5.37
3	Prathiba & Sarah Kuruvilla	o Well o Poor		4.2 5.3
4	Seema Kashyap & Kusum Kapila	o Well o Mod o Poor	2.17-7.52 2.24-4.68 3.01-3.89	3.66 3.04 3.45
5	Miller et al	o Well o Poor		2.9 4.0
6	Present Study	o Well o Mod o Poor		3.76 3.84 4.12

Our study correlates well with study conducted by Ghalaza Mehdi(18), Prathiba(39) and Miller et al(29) that the count increases when the neoplasm becomes well to poorly differentiated.

However in contrast Jyotima Agarwal(17) reported the AgNOR count is high in moderately differentiated squamous cell carcinoma followed by poorly differentiated squamous cell carcinoma. Likewise Seema Kashyap et al(19,46) reported high AgNOR count in well differentiated followed by poorly differentiated and moderately

differentiated squamous cell carcinoma.

Rowland's (1988) in a study of AgNOR in Cervical Intraepithelial Neoplasia did not give any significant difference in AgNOR counts in normal squamous epithelium CIN I and CIN II, but there was a significant increase in CIN III group as well as invasive cancer(10,11).

Crocker et al who have done intensive work on NOR's in various tumors have observed three main types of AgNOR configurations in normal neoplastic cells(6,7,811,12). All three types of NOR patterns were observed in his study

- The NOR's are fully aggregated to form a solitary rounded structure, often seen in resting cell.

- Nucleolar pattern in proliferating cells, where NOR's can be seen within the nucleolus

- Dispersion of small NOR's throughout the nucleoplasm as frequently observed in highly malignant cells.

Our study correlates with the other studies and literature that AgNOR count gradually increases from the normal to invasive cancer. In our study, the 25 control cases show an AgNOR count/cell of 1-3 with a mean of 1.63 and 3 cases exhibit a size variation of 2+ to 3+ and distribution variation of 2+ to 3+ in 2 cases. This may be reflected as initial SIL changes / Koilocytic changes / as a response to intense

inflammatory reaction.

These cases are subjected for repeat Pap smears at 3 month intervals, revealed decreased in AgNOR counts to normal ratio at the end of one year.

With the standardization of the Silver staining technique diagnostic pathology has achieved a new milestone. The AgNOR's have been shown to reflect DNA transcriptional activity. Study of AgNOR's has been identified as a reliable indicator of cell proliferation and in turn of the malignant potential of a lesion.

Malignant tumor cells are characterized by extremely large AgNOR's which show a random or scattered distribution. They are useful in discriminating between benign and malignant conditions being significantly higher in malignant cells than in normal cells. They also serve as a significant prognostic indicator in malignant lesions.

TABLE -8

Sl.No	Grade	Prior RT AgNOR count/cell		4 weeks after RT AgNOR count/cell		8 weeks after RT AgNOR count/cell	
		Range	Mean	Range	Mean	Range	Mean
1	Well differentiated squamous cell carcinoma	3-8	3.76	2-6	3.2	2-5	2.67
2	Moderately differentiated squamous cell carcinoma	3-5	3.84	2-4	2.82	2-3	2.19
3	Poorly differentiated squamous cell carcinoma	3-9	4.12	2-5	3.35	2-4	2.97

The *Table-8* shows in our prospective study, the AgNOR count/cell has

reduced from 3.76 to 3.2 and 2.67 (after 4 weeks and 8 weeks of radiotherapy in Well differentiated squamous cell carcinoma. However in Moderately differentiated squamous cell carcinoma the reduction is so impressive from a mean of 3.84 to 2.82 to 2.19 (after 4 weeks and 8 weeks of Radiotherapy).

Poorly differentiated squamous cell carcinoma also shows a reduction from 4.12 to 3.35 and 2.97. Our study correlates well with Ghazala Mehdi(30) that Moderately differentiated squamous cell carcinoma responds well with Radiotherapy followed by Well differentiated squamous cell carcinoma. Poorly differentiated squamous cell carcinoma even though shows reduction in AgNOR range in the mean, prior and post radiation, there is clear evidence that these cancers responds poorly to external radiotherapy alone, which suggests the use of internal as well as external Radiotherapy in combination in cases of Poorly as well as Well differentiated squamous cell carcinoma.

In our study 8 cases shows persistence of malignancy after 8 weeks of radiotherapy as given in the following *Table-9*.

TABLE-9

Sl. No	Grade of Tumor	Persistence of malignancy No. of cases	Size of Tumor	Stage	Anaemia Grade	Type of Radiotherapy		Disrupted Treatment	
						External RT	Internal RT	8 wks	> 8 wks
1	Well Differentiated Squamous cell carcinoma	4	> 7cm	III B III B III A IV A	+++ ++ +++ +++	√ √ √ √	x x x x	√ - - -	- √ √ √
2	Moderately Differentiated Squamous cell carcinoma	2	> 6cm	IV A IV A	+++ +++	√ √	x x	- √	√ -
3	Poorly Differentiated Squamous cell carcinoma	2	> 7cm	IV A IV A	+++ +++	√ √	x x	- -	√ √

All cases with recurrence exhibit advanced stage at the time of presentation (>III A / III B) and severely anaemic, all cases were treated only with external Radiotherapy and they did not stick on to the treatment modality.

CONCLUSION

In the present prospective study comprising of 50 Papanicoloau cervical smears of carcinoma cervix and 25 controls suggest the following conclusions.

1. The incidence of cervical cancers in semi urban areas – Thanjavur is 51/1, 00,000 population.
2. Cervical cancers are common between the age group of 41-50 years which is in contrast low compared to western population where it is common in 51-60 years.
3. **Squamous cell carcinoma of cervix** constitutes **80% of the gynecological cancers.**
4. **Most patients** presents **initially at an advanced stage (i.e., > II B).**
5. **Moderately differentiated squamous cell carcinoma** is the **commonest** type observed as in other.
6. AgNOR technique provides an index of cell proliferation.
7. AgNOR count/cell increases gradually from normal to SIL changes to invasive carcinomas.
8. The AgNOR count is **high in Poorly differentiated carcinomas** and **low in Well differentiated carcinomas.**
9. Moderately differentiated squamous cell carcinoma responds well to external radiotherapy alone.
10. Poorly differentiated squamous cell carcinoma requires both external and internal radiation for prognosis.
11. Viral induced changes can impart a slight increase in AgNOR count in normal individuals.
12. AgNOR study provides a **standard method to evaluate prognosis of the patient after radiotherapy.**

The AgNOR technique which was used extensively in cytogenetics earlier, has now gained importance as an indicator of cell proliferation. In normal cells, the AgNOR are tightly packed in the nucleoli and are indiscernible. In rapidly proliferating neoplastic cells nucleolar disaggregation takes place resulting in dispersion of individual AgNOR's.

So AgNOR count is a **reproducible, simple, efficient and inexpensive method** which can be used as an **adjunct to routine cytology for diagnosis of cervical carcinoma especially in doubtful cases and also used as a prognostic indicator.**

APPENDIX-1

AgNOR STAINING

PREPARATION OF STAINING SOLUTION

Solution A: 2% gelatin in 100 ml of 1% Formic acid

Solution B: 50% aqueous silver nitrate solution in de-ionized water

WORKING SOLUTION

One part of Solution A mixed with two parts of Solution B

STAINING METHOD

The wet fixed cervical smears were exposed to freshly prepared working solution for 30 min at 37°c, left in dark.

Slides were dehydrated in 3 changes of acetone, cleared in Xylene and
mounted in DPX.

APPENDIX-2

PAPANICOLAOU STAINING METHOD

7) Wet fixed smear, rinse in water for 1 min
8) Stain in Harris Haemotoxylin , 5 min
9) Rinse in water, 2 min
10) Differentiate in 0.5% aqueous hydrochloric acid, 10 seconds approx
11) Rinse in water , 2 min
12) " BLUE " in Scott's tap water substitute, 2 min
13) Rinse in water, 2 min
14) Dehydrate, 70 percent alcohol for 2 min
15) Dehydrate, 95 percent alcohol for 2 min
16) Dehydrate, 95 percent alcohol for 2 min
17) Stain in OG 6, 2 min
18) Rinse in 95 percent alcohol, 2 min
19) Rinse in 95 percent alcohol, 2 min
20) Stain in EA 50, 3 min
21) Rinse in 95 percent alcohol, 1 min

From

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